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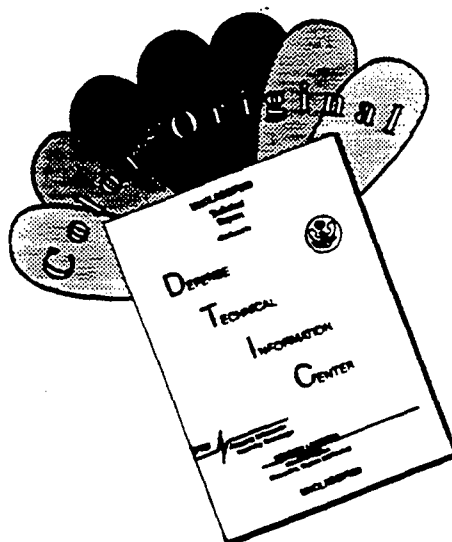
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13. ABSTRACT (Maximum 200
Research indicates that neuropeptide hormones (vasopressin, oxytocin) are produced by breast cancer cells. Benign fibrocystic breast disease however does not appear to express the vasopressin gene. Such results indicate that expression of neuropeptide genes may represent part of the carcinogenic process. The interaction of neuropeptide through autocrine/paracrine/endocrine mechanisms with receptors on breast cancer cells represents a way in which these peptides might influence cancer cell pathophysiology. However, the expression of neuropeptide receptors and the evoked signaling cascades in breast cancer cells have not been thoroughly examined. An RT-PCR approach, using primers designed to amplify the specific vasopressin receptors and oxytocin receptor, has been implemented. Using this approach, PCR products for VACM, V1b, and V2 vasopressin receptors, and the oxytocin receptor have been amplified from breast cancer cells. Vasopressin V1a receptor expression was not demonstrated using RT-PCR. Using Northern blot and a probe against human VACM, 3 mRNAs (~3.5,5,6.5Kb) were detected. In MCF-7 cells, vasopressin and a V1 agonist induced tyrosine phorphory-lation of MAPK. The results indicate that breast cancer cells express neuropeptide receptors and that activation of these receptors can stimulate signaling events associated with cancer cell growth.

14. SUBJECT TERMS Breast Cancer, Anatomical Samples, Vasopressin, Vasopressin-Receptor, Growth Factor, Cell-Surface Antigen, Early Detection, Treatment

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(4) TABLE OF CONTENTS

(5) INTRODUCTION

Vasopressin is a nine amino acid hormone which is produced by neurons in two distinct regions of the hypothalamus called the paraventricular nucleus and the supraoptic nucleus. Research indicates that neuropeptide hormones like vasopressin can also be produced by certain types of cancer such as small cell-lung carcinoma and breast cancer (Gupta et al., 1986; North, 1991; Howard et al., 1993; Friedmann et al., 1994; North et al., 1995). In contrast, vasopressin-gene expression does not appear to be a common feature of benign fibrocystic disease, thus suggesting that expression of the vasopressin gene may be part of the carcinogenic process (Fay et al., unpublished data, see appendix for prepublication). The production, secretion, and interaction of hormone with cell surface vasopressin receptors represents a possible autocrine/paracrine role for this peptide in cancer cells. In support of this hypothesis vasopressin was shown to have a growth-promoting influence on MCF-7 breast cancer cells (Taylor et al, 1990), and to induce a rise in intracellular free calcium in a cultured breast cancer cell line (Bunn et al., 1992; Fay et al., unpublished data). Also, in a transgenic mouse model of breast cancer, ectopic vasopressin was found to stimulate cancer growth, but did not influence the time to tumor onset (Chooi et al., 1994).

Although it appears that vasopressin is being produced by cancer cells and is influencing cancer cell growth, the receptor(s) through which vasopressin is having an influence have not been thoroughly documented. Four cell surface vasopressin receptors have been cloned, (Birnbaumer et al., 1992; Hirasawa et al., 1994; Sugiomoto et al., 1994; Thibonnier et al., 1994; Burnatowska-Hledin et al., 1995). These include the V1a and V1b vasopressin receptors, which are involved with vasoconstriction and the release of ACTH, respectively. The transduction cascade associated with the V1 receptors involves the activation of a phospholipase, with the subsequent production of inositol 1,4,5-triphosphate, 1,2-diacylglycerol, calcium mobilization and influx, and protein kinase C activation. The V2 vasopressin receptor is located in the tubules of the kidney, and is responsible for the reabsorption of water. The V2 receptor transduction cascade involves the activation of adenylate cyclase, leading to an increase in cAMP, and the activation of protein kinase A. In addition, a novel calcium-mobilizing vasopressin receptor (VACM-1) has been cloned and characterized from rabbit kidney (Burnatowska-Hledin et al., 1995). This novel receptor is unique in that it appears to have only one transmembrane domain as compared to the other vasopressin receptors which have 7-transmembrane domains. Several studies indicate that a cell line derived from a dimethylbezan(a)anthracene-induced rat mammary tumor possesses functional V1a vasopressin receptors (Monaco et al., 1978; Monaco et al., 1980; Guillon et al., 1986; Kirk et al., 1986; Woods

and Monaco, 1988). Using binding of radiolabeled vasopressin it was shown the MCF-7 breast cancer cells express V1 vasopressin receptors (Taylor et al., 1990). However, no thorough analysis of vasopressin receptor expression in breast cancer has been performed.

In addition to this, it has recently been published that up to 91% of breast cancer cells express oxytocin receptor protein (Ito et al., 1996), and that activation of this receptor by oxytocin may modulate breast cancer cell growth (Taylor et al., 1990; Cassoni et al., 1994). Since we found that 44% of breast cancer examined express oxytocin immunoreactivity (North et al., 1995) and that oxytocin can stimulate tyrosine phosphorylation in cultured breast cancer cells (see last progress report) we have also commenced an effort to examine oxytocin receptor expression in cultured breast cancer cells.

In this progress report studies are presented aimed at examining the expression of vasopressin, vasopressin receptors, and oxytocin receptors in cultured breast cancer cells. In addition, studies are presented aimed at elucidating what signaling events neuropeptides like vasopressin activate in breast cancer cells and how these relate to cancer cell pathophysiology. During the final year of this postdoctoral fellowship I will continue the studies aimed at 1) evaluating vasopressin production by breast cancer 2) determining what neuropeptide receptors are expressed by breast cancer cells, and 3) elucidating the intracellular signaling events that are activated and how these relate to cancer cell pathophysiology.

(6) BODY

A. Vasopressin gene-related products in fibrocystic breast disease. As mentioned in the last progress report we have completed a survey of the expression of vasopressin gene-related products in fibrocystic breast disease using the technique of immunohistochemistry and antibodies directed against vasopressin and vasopressin-associated glycopeptide. These studies address the proposed work in technical objective 1 of the original grant application. This work has been submitted to the British Journal of Cancer for publication as a short communication. For a detailed account of methodology, results, and discussion please refer to the copy of the submitted manuscript in the appendix. The results of these studies indicate that various cases of benign fibrocystic breast disease do not express vasopressin gene-related products as determined by the method of immunohistochemistry. These results indicate that expression of vasopressin gene-related products does not appear to occur in benign breast lesions, and that expression of vasopressin gene-related products is not just a marker of cellular proliferation in the breast. We

are currently examining cases of carcinoma in situ to determine if vasopressin expression occurs in pre-metastatic breast lesions.

B. <u>Reverse transcription polymerase chain reaction for vasopressin, and vasopressin and oxytocin receptors in cultured breast cancer cells.</u>

Using the technique of RT-PCR (reverse transcription polymerase chain reaction) and primers specific for vasopressin V1a receptor, V1b receptor, V2 receptor, the recently cloned vasopressin-activated calcium mobilizing receptor (VACM), and the OT receptor (see Table 1 below for sequence of the primer pairs) we have initiated an investigation of the expression of these mRNAs in cultured breast cancer cells. These studies specifically address technical objective 3 of the original grant application, and represent unpublished data. An RT-PCR approach was initiated instead of a radioligand approach because it was anticipated that possibly more than one vasopressin receptor mRNA was being expressed by breast cancer cells. In addition, an examination of vasopressin mRNA has been initiated using primers based on the human vasopressin cDNA. Examining vasopressin expression in breast cancer cells addresses Technical Objective 2 of the original grant application. The basic protocol used for RT-PCR is as follows. Poly(A)+ RNA was isolated directly from cultured breast cancer cell lines (BT 549, MCF-7, MDA-MB-231, T47D, ZR-75) using oligo(dT) cellulose as described by Badley et al. (1988). Briefly, subconfluent cultures were lysed in a buffer consisting of 0.2M NaCl, 0.2M Tris- HCl, 1.5mM $MgCl_2$, 2% SDS, and 200 µg/ml proteinase K. Lysates were passed through 18 and 23 gauge needles to shear DNA, and incubated at 45 ˚C for 1.5 hrs in a shaker water bath. The NaCl concentration of the samples was brought up to 0.5 M and ~0.2g of oligo(dT) cellulose (Boehringer Mannheim) was added to each sample. The samples were incubated at room temperature for 30 min with gentle rotation. Cellulose was pelleted (500 rpm) and samples washed 3x with binding buffer (0.5 M NaCl, 0.01 M Tris-HCl pH 7.5). Samples were resuspended in ~10 ml of binding buffer and samples placed onto Biorad poly-prep columns. The columns were allowed to run dry and washed 1X with an additional 10 ml of binding buffer. RNA was eluted from the columns using elution buffer (0.01 M Tris-HCl pH 7.5) and brief centrifugation of the columns (500 rpm). The RNA was precipitated overnight at -20 ˚C using 0.1 vol of 3 M Na-acetate and 2 vol of 100% ethanol. RNA was pelleted by centrifugation (30 min, 14,000 x g, 4 ˚C), and washed with 70% ethanol. The RNA was resuspended in DEPC treated water, and quantified by measuring the absorbance at 260 nm. Samples were stored at -80 ˚C until used. RT-PCR was performed using the Gene AMP® kit and the primer pairs shown in Table 1. First strand cDNA synthesis was performed using 4µg of poly(A)+ in a mixture (20 µl total volume) containing 5 mM $MgCl_2$, 1X PCR buffer II, 1mM of each dNTP (dGTP, dATP, dTTP, dCTP), 1U/µl RNase

inhibitor, 2.5U/µl MuLV reverse transcriptase, and 2.5µM Oligo d(T). The conditions for RT were 10 min at rm temp and 15 min at 42 ˚C. The PCR mixture (50 µl total volume) consisted of 10µl of the RT, 200 µM of each primer, 2 mM MgCl2, 1X PCR buffer II, and1.25 U of AmpliTaq® DNA polymerase. PCR was performed using an ERICOMP EasyCycler™ and the following conditions, 97 ˚C for 8 min, and 30 cycles that included 30 sec at 95 ˚C, 1 min 30 sec at 58 ˚C, 1 min 30 sec at 72 ˚C, and a final extension step at 72 ˚C for 10 min. PCR products were electrophoresed for 1 hr at 100 volts using a 2% agarose gel and TAE buffer. A 100 bp ladder was used to determine the size of amplified products and the gels were stained with ethidium bromide to visualize the DNA. As shown in Fig 1 the 391 bp human oxytocin receptor PCR product was amplified from all 5 breast cancer cell lines examined. In addition we have amplified the oxytocin receptor PCR product from the MCF-7 cell line using as little as 2 µg of total RNA. These results suggest that the human oxytocin receptor mRNA is expressed by cultured human breast cancer cell lines. These results confirm the studies of Cassoni et al. (1994) who demonstrated oxytocin receptor mRNA in several cultured human breast cancer cell lines using a similar RT-PCR approach, and Taylor et al (1990) who showed oxytocin receptor protein production in MCF-7 cells using the technique of radioligand binding. In addition, we have been able to amplify the 193 bp and the 674 bp VACM receptor PCR products from the MCF-7 , ZR-75, and T47D breast cancer cells (see Fig 2). Predicted size products for the V1b and V2 vasopressin receptors were amplified from MCF-7 cells (data not shown). A PCR product of the predicted size (313 bp) for vasopressin was amplified from the MCF-7, T47D, and Zr-75 cell lines (data not shown).

TABLE 1.
Primer Sequences (5' to 3')
a. Oxytocin receptor primers
forward primer 5'-CCTTCATCGTGTGGCTGGAC G-3'
reverse primer 5'-CTAGGAGCAGAGCACTTATG-3'.
The oxytocin receptor primer pair amplify a PCR product of 391 base pairs.

b. Vasopressin V1a receptor primers
forward primer (bp 939-958) 5'-TGTGTCAGCAGCGTGAAGTC-3'
reverse primer (bp 1325-1346) 5'-GGACTTGGAAGATTTAGGCGAG-3'
The V1a primers amplify a 408 base pair PCR product.

c. Vasopressin V1b receptor primers
forward primer (bp 1154-1173) 5'-CCAATGTGGCTTTCACCATC-3'
reverse primer (bp 1372-1392) 5'-TAGGCTGAGGCTGAGGCTGAG-3'

The V1b receptor primers amplify a 239 bp PCR product.

d. Vasopressin-activated calcium mobilizing receptor primers (VACM)
forward primer (bp 1825-1848) 5'-GAATGGCTAAGAGAAGTTGGTATG-3'
reverse primer (bp 2475-2498) 5'-TCTTCTCTCATCCTTTCTGTAGTG-3'
The VACM primer pair amplify a PCR product of 674 base pairs.

e. Vasopressin-activated calcium mobilizing receptor primers (VACM)
forward primer (bp 1679 -1702) 5'-CACCATTAAGCAAAACTACCTCTG-3'
reverse primer (bp 1802 - 1825) 5'-CATACCAACTTCTCTTAGCCACTC-3'
This VACM primer pair amplify a PCR product of 193 base pairs.

f. Vasopressin primers
forward primer (bp 120 -140) 5'-ATGTCCGACCTGGAGCTGAGA-3'
reverse primer (bp 411- 432) 5'-CGTCCAGCTGCGTGGCGTTGCT-3'
The vasopressin primers amplify a PCR product of 313 base pairs.
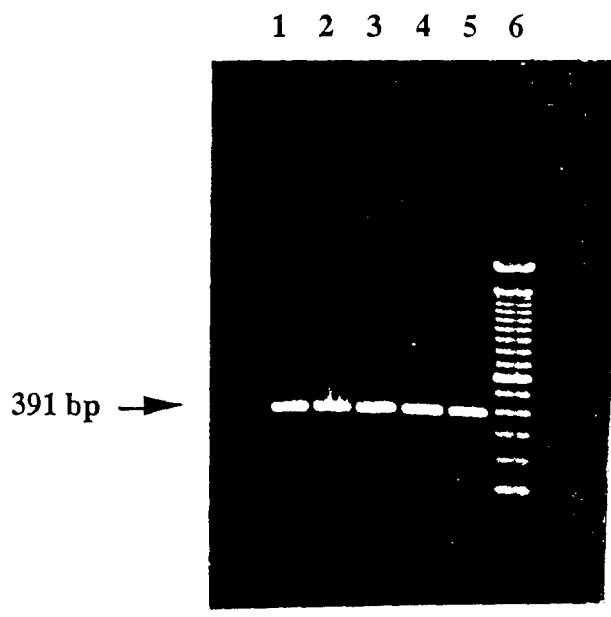
Fig 1   Demonstration of oxytocin receptor mRNA in human breast cancer cell lines using RT-PCR and primers specific for the human oxytocin receptor.   The 391 bp human oxytocin receptor PCR product was amplified from all 5 breast cancer cell lines.   Lane 1, BT549 cell line; Lane 2, MCF7 cell line; lane 3, MDA-MB-231 cell line; Lane 4, T47D cell line; Lane 5, ZR-75 cell line; Lane 6, 100 bp DNA ladder.
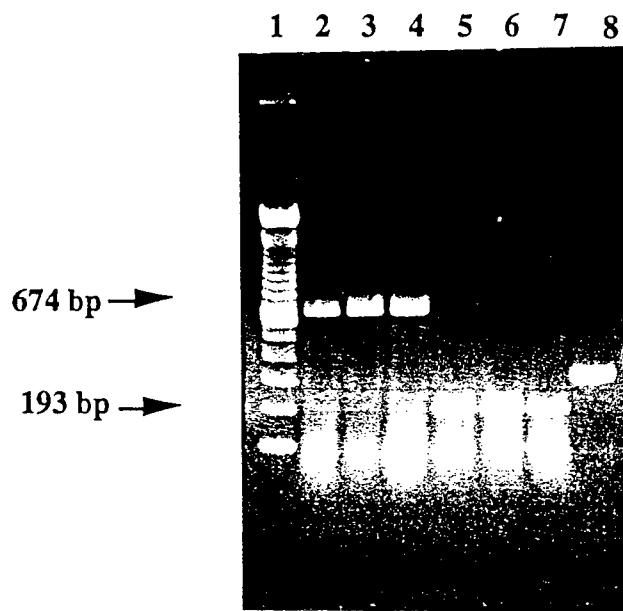
Fig 2   Demonstration of VACM receptor in human breast cancer cells using RT-PCR and primers based on the sequence for the cloned rabbit VACM receptor.   The 674 bp PCR product was amplified from the MCF-7, T47D and ZR-75 breast cancer cells (lanes 2-4, left to right).   The 193 bp PCR product was amplified from MCF-7, T47D, and Zr-75 breast cancer cells (lanes 5-7, left to right).   Lane 1 is the 100 bp DNA ladder and lane 8 is the PCR positive control.

C. Direct sequencing of PCR products

This is unpublished data.   PCR products were reamplified until a strong single band was apparent.   The bands where excised from the agarose gel and the products purified using millipore spin columns and Princeton Separation Centri Sep spin columns.   Direct Sequencing was performed using 60 to 70 ng of PCR product, Amplitaq® DNA polymerase, the Taq Dyedeoxy™ terminator cycle sequencing kit (Applied Biosystems), and a Model 373A Applied Biosystems automated DNA sequencer.   The primers

used for sequencing were the forward primers described above. Analysis of the DNA sequence data was performed using the BLAST network service (Altschul et al., 1990) We have used this technique to sequence the VACM PCR products from MCF-7 cells, and the DNA sequence obtained has a high degree of identity to the published rabbit VACM sequence (Data not shown). This represents the first sequence data obtained for human VACM.Sequencing of the V1b PCR product from MCF-7 cells verified that this PCR product was amplified from V1b mRNA in these cells (Data not shown). The vasopressin product amplified from ZR-75 breast cancer cells had only partial identity to vasopressin cDNA. We are currently trying to identify what this amplified product is, and are designing additional primer pairs to determine if the cultured breast cancer cells also express normal vasopressin mRNA.

D. Northern blot analysis of VACM receptor in breast cancer cells
This is unpublished data. Briefly, RNA (5-8 ug) from Zr-75, MCF-7 and T47d breast cancer cells was electrophoresed on a 1.2% agarose formaldehyde denaturing gel for 3-4 hrs at 75 volts using 1X MOPS (3-(N-morpholino)propanesulfonic acid), and transferred overnight to a supported nitrocellulose membrane using 20X SSC. Membranes were baked (80°C) for 2 hrs in a vacuum oven, and prehybridized in hybridization solution consisting of 5x SSC, 5x Denhardt's solution (0.1% (w/v) ficoll type 400, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin), 200 μg/ml denatured salmon sperm DNA, 0.1% SDS, 6.25 mM NaH2PO4 (pH 6.5) and 50% formamide. The 674 bp VACM PCR product was randomly labelled with [^{32}P] using exonuclease free Klenow fragment and the Ambion DECAprime II™ labeling Kit. Approximately 10^6 CPM of the labeled probe per ml of hybridization solution (same as prehyb. solution) was used for the Northern. Hybridization was performed overnight at 42 °C. The membrane was washed 1 x 30 sec with 2x SSC/0.1%SDS (room temp), 2 x 15 min with 2xSSC/0.1% SDS (rm temp), and 2x with 0.1X SSC/0.1% SDS (37 °C). The washed membrane was wrapped in plastic and exposed to x-ray film with intensifying screens at -70 °C for approximately 1 week. The results obtained indicate three distinct bands at approximate sizes of 3.5, 5. and 6.5 Kb (data not shown). At this stage it is not clear if these multiple bands represent precursor mRNA and degradation products, or multiple isoforms. Thus PCR and Northern analysis support the expression of VACM receptor mRNA in human breast cancer cells. As an auxiliary project the lab is currently attempting to clone the human VACM receptor. These results are being presented as part of a poster at the 1996 annual meeting of the American Society for Cell Biology (see abstract in Appendix).

E. Neuropeptide-induced tyrosine phosphorylation of mitogen-activated protein kinase in MCF-7 cultured breast cancer cells.

These results are not published. Taylor et al., (1990) have previously demonstrated that vasopressin is capable of stimulating the growth of MCF-7 breast cancer cells in vitro, and Chooi et al., (1994) have shown that vasopressin stimulates breast tumor growth in vivo. However, the mechanisms by which vasopressin is exerting these effects, and the receptors involved remain uncharacterized. One common pathway for growth factor action is stimulation of the mitogen activated protein kinase (MAPK) cascade. Activation of MAPK occurs by phosphorylation by MAP kinase kinase of first a tyrosine residue followed by a threonine residue on MAPK. Using recently available antibodies from New England Biolabs which are directed against tyrosine-phosphorylated MAPK we have initiated an investigation of vasopressin induced activation of the MAPK cascade in MCF-7 breast cancer cells. These studies address the aims of Technical objective 3 of the original grant application. For these experiments 2×10^6 cells were seeded into 6-well plates in medium containing 0.5% FBS. After 2 days, the medium was aspirated and replaced with new medium to remove endogenous growth factors. Cells were then treated with medium with 0.5% FBS (media control), media containing vasopressin (100, 1000 nM), media containing V1 receptor agonist ([Phe^2, Orn^8]-Oxytocin; 300, 3,000 nM), or 20% FBS (positive control) for 1 hr at 37 °C. The cells were washed with PBS, and lysed in boiling SDS sample buffer. A aliquot of each sample was subjected to SDS-PAGE, and the proteins transferred to a PVDF membrane. Membranes were blocked using 5% BSA in PBS for 1 hr at room temperature. The membranes were then incubated with the anti-phospho MAPK antibody (1:1,000 dilution) overnight at 4°C. Protein bands were detected using a alkaline phosphatase conjugated anti-rabbit antibody and the CDP-Star™ chemiluminescent reagent (New England Biolabs). The membranes were then exposed to x-ray film for 5 minutes. As shown in figure 3 vasopressin, the vasopressin V1 agonist, and 20% FBS appeared to induce an increase in tyrosine phosphorylated MAPK as compared to the media control. These results indicate that vasopressin may be inducing signaling events in breast cancer cells that could increase cancer cell growth.
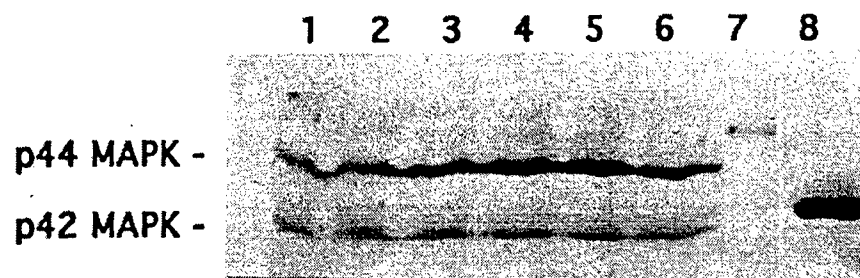
Fig 4. Vasopressin induced tyrosine phosphorylation of MAP kinase in MCF7 breast cancer cells. Cells were treated with vasopressin (100, 1,000 nM) or a V1 vasopressin receptor agonist ([Phe^2, Orn^8]-oxytocin, 300 nM, 3,000 nM) for 1 hr. Lane 1, 0 mM vasopressin; Lane 2, 100 nM Vasopressin; Lane 3, 1,000 nM vasopressin; Lane 4, 300 nM V1 agonist; Lane 5, 3,000 nM V1 agonist; lane 6, cell treated with 20% FBS (positive control); Lane 7, non-phosphorylated p42 MAP kinase control; Lane 8, phosphorylated p42 MAP kinase control.

F. Vasopressin-induced calcium mobilization.

As introduced in the last progress report we have determined that vasopressin induces calcium mobilization in ZR-75 and T47D breast cancer cells. One interesting observation from these studies is that in both of these cases only a subpopulation of the cell sample appear to respond to hormone (10 - 25%). This raises the possibility of heterogeneous expression of vasopressin receptors within the cell population or the possibility of differential susceptibility to vasopressin at different stages of the cell cycle. To examine vasopressin-induced calcium mobilization on a cell to cell basis, I have developed a microscopic technique using a Meridian Ultima confocal microscope (A shared facility of the Cell Analysis Laboratory at Dartmouth Medical School). For this method cells are plated onto glass chamber slides (Nunc). the day before experimentation the cells placed in serum-free medium containing 0.1% BSA. Cells are washed 2x with serum-free medium, and incubated for 1 hr with serum-free medium containing 10 μM Indo-1 AM and 1 μl/ml of pluronic (0.2 mg/ml stock). After incubation the cells are washed 2x with physiological saline solution (118 mM NaCl, 5 mM KCl, 1.6 mM CaCl2, 1.2 mM MgCl2, 1.2 mM Na2HPO4, 100 mM glucose, 0.1% BSA), and cells are placed on ice in physiological saline solution. For experimentation, cells are prewarmed to 37 °C on the stage of the microscope. When a suitable cell is found, several prescans are made [excitation is at 356 nm, and emission monitored at 405nm (calcium-

bound Indo), and at 485 nm (unbound Indo)]. At the time of treatment 0.5 ml of 2x the desired concentration of vasopressin is added to the well. To optimize assay conditions I have used A10 vascular smooth muscle cells, which are known to express V1a vasopressin receptors, and exhibit vasopressin-induced calcium mobilization and influx. As shown by the glossy print in the appendix, when 1,000 nM vasopressin is added to the vascular smooth muscle cells at 40 sec, there is a rapid increase in intracellular free calcium as indicated by the red, yellow and white pixels. Note that intracellular-free calcium begins to return to prestimulation values, indicative of a physiological response. The line graph on this glossy print indicates the ratio of the 405nm/485nm, and depicts the rise in intracellular free calcium, and the return to baseline calcium levels. I have attempted to use this method to examine vasopressin-induced calcium mobilization in the ZR-75 breast cells, which we have previously shown to exhibit vasopressin-induced calcium mobilization by flow cytometry, However, the ZR-75 cells did not stay attached to the glass coverslip during the rigorous loading and washing procedure. I have attempted to use this technique to examine vasopressin-induced calcium mobilization in 2 rat mammary carcinoma cell lines (NMU and RBA), which exhibit very good adherence to plastic and glass as exhibited by resistance to trypsinization. However, in these 2 cell lines vasopressin (100, 1,000 nM) and a vasopressin V1 agonist (100, 1,000 nM) had no influence on intracellular-free calcium (data not shown). I am in the process of attempting to coat the glass chambers with extracellular matrix (e.g. matrigel), to try and promote the adherence of ZR-75 cells so that they can be examined using this technique.

G. Vasopressin-induced generation of inositol phosphate in breast cancer cells .

To study vasopressin-stimulated accumulation of inositol phosphates in breast cancer cells an assay for measuring the production of inositol phosphates has been developed. These studies address the work proposed in Technical Objective 3 of the original grant application. These experiments were initiated the week prior to preparation of this progress report and the samples have not yet been processed for counting yet. ZR-75 breast cancer cells at ~75 % confluency in 60 x 15 mm plates were incubated for 40 hrs in RPMI-1640 growth media lacking myo-inositol supplemented with 0.1% BSA and 5 µCi/ml [^{3}H]myo-inositol. The media was aspirated and the cells were removed from the plates using non-enzymatic dissociation solution. Approximately 10^6 cells per ml were stimulated with 1,000 nM vasopressin dissolved in physiological saline solution (105 mM NaCl, 30 mM LiCl, 4.2 mM NaHCO$_3$, 5.9 mM KCl, 1.8 mM

CaCl$_2$, 1.4 mM MgCl$_2$, 1.2 mM NaH$_2$PO$_4$, 11 mM glucose, 10 mM HEPES; pH 7.35) for 15 and 30 min. Control samples for each time point received just the physiological saline-lithium chloride solution. After the allotted time the cells were extracted with 5N perchloric acid (50 μl) containing 1 mM EDTA and 5 mM DEPTA. The extracted samples were placed on ice for 30 min and 1.5% K$_2$CO$_3$ added to precipitate cellular debris. The samples were then pelleted and the supernatants neutralized with 10N NaOH. Total inositols were isolated using an anion exchange resin (1:10, AG1-X8, formate form) packed into small columns (1 ml packed resin). Columns are initially washed sequentially with 4 ml of 2M ammonium formate and 0.1 M formic acid, 4 ml of ddH$_2$O, and 4 ml of 20 mM NH$_4$OH. The samples were loaded onto the column, and the columns washed with 3 ml of 40 mM NH$_4$OH, 4 ml of 40 mM ammonium formate, and finally with 4 ml of 2M ammonium formate with 0.1 M formic acid to purge the total inositols from the column.

H. Ionic currents in T47D human breast cancer cells

Although not directly related to the technical objectives of the original grant, we have initiated a collaborative breast cancer project with Dr. Frances McCann of the Physiology Department at Dartmouth Medical School. The goal of this project was to identify the ion currents present in breast cancer cells since ionic signaling events have been linked to mitogenesis (Dubois, J.M. and Rouzaire-Dubois, B., 1993). Using the whole cell configuration of the patch clamp technique we have identified a chloride current and a voltage-gated potassium current in T47D human breast cancer cells. For a detailed account of the methods, results and discussion of this work please see the reprint in the appendix (Gallagher et al., 1996). With Dr. McCann we are also examining if vasopressin and oxytocin induce any ion currents in breast cancer cells.

(7) CONCLUSIONS

Vasopressin has been shown to stimulate breast cancer cell growth in vitro (Taylor et al., 1990) and in vivo (Chooi et al., 1994). However, the receptors and the signaling transduction events mediating these growth effects have not been documented. Using RT-PCR we have been able to amplify PCR products from cultured breast cancer cells for VACM-1, V1b, and V2 vasopressin receptors, and for the oxytocin receptor. The authenticity of the VACM-1 and V1b PCR products have been confirmed by DNA sequencing. These results suggest that the vasopressin-induced calcium mobilization that we have observed in ZR-75 and T47D breast cancer cells (see last progress report) could result from activation of VACM-1 or V1b receptors. Other researchers have demonstrated V1a

vasopressin receptor expression by a cell line derived from a dimethylbezan(a)anthracene-induced rat mammary tumor (Monaco et al., 1978; Monaco et al., 1980; Guillon, et al., 1986; Kirk et al., 1986; Woods and Monaco, 1988). In contrast to this finding we have been unable to amplify V1a receptor mRNA from MCF-7 human breast cancer cells by RT-PCR, and have been unable to demonstrate vasopressin-induced calcium mobilization in a N-nitroso-methyurea induced rat mammary adenocarcinoma cell line (NMU), and in a 7,12-dimethyl-benz[a]anthracene induced rat mammary adenocarcinoma cell line (RBA). Additional primers to the V1a vasopressin receptor are being designed to verify these results.

To verify the RT-PCR data which show that cultured human breast cancer cells express VACM-1 vasopressin receptor mRNA we have performed a Northern analysis using the 674 bp VACM-1 PCR product as a probe. Using this approach 3 mRNA species were identified with approximate sizes of 3.5, 5, and 6.5 Kb. At this time it is not known if these different sized mRNAs represent precursor mRNA and degradation products or multiple isoforms of VACM-1. The laboratory is currently attempting to clone human VACM-1 in an attempt to further elucidate the role of this novel vasopressin receptor in breast cancer pathophysiology.

In confirmation of published studies which shows that up to 91% of breast cancer specimens express oxytocin receptors, (Bussolati et al., 1996; et al., 1996), we have demonstrated oxytocin receptor mRNA expression in all of 5 cultured breast cancer cell lines examined. Like vasopressin, several studies indicate that oxytocin may also modulate breast cancer cell growth (Cassoni et al., 1994; Cassoni et al., 1996), and previously we demonstrated that oxytocin induced tyrosine phosphorylation of a number of proteins in cultured MCF-7 breast cancer cells (see last progress report). To further evaluate the role of activated oxytocin receptors in breast cancer this principle investigator has submitted a career development award to the Department of defense.

Previously, we demonstrated, using immunohistochemistry and antibodies to different regions of the vasopressin precursor, that expression of vasopressin gene-related products is a common feature of breast cancer cells (North et al., 1995). Also, there are two reports of patients with breast carcinoma presenting with the syndrome of Inappropriate antidiuretic hormone (vasopressin) secretion (Gupta et al., 1986; Howard et al., 1993) These results support an autocrine/paracrine role for this peptide in cancer cell pathophysiology. To further these studies we have initiated an RT-PCR approach to examine vasopressin gene expression in cultured breast cancer cells. Using primers designed against vasopressin mRNA we amplified a product which appeared to be of the predicted size. However, DNA sequencing of this product revealed only partial identity to the vasopressin cDNA sequence. Currently the identity

of this PCR product is under investigation, and additional primer pairs are being designed to determine if cultured breast cancer cells express normal human vasopressin mRNA.

Mitogen activated protein kinases (ERK1 and ERK2) are activated by a number of growth factors and protooncogene products (for reviews see Davis, 1993; L'Allemain, 1994; Cobb and Goldsmith, 1995). Activation of the MAP kinase cascade classically involves the activation of a receptor tyrosine kinase after binding of ligand. This, in turn, results in autophosphorylation on tyrosine residues and the binding to adaptors (Grb2) through SH2 domains. These adaptor proteins then recruit guanine nucleotide exchange factors through proline rich SH3 domains, and the exchange factors promote Ras to associate with GTP. The activated GTP-bound Ras then targets Raf to the membrane, which phosphorylates and activates a MAP kinase kinase (MEKS). These MAP kinase kinases in turn phosphorylate and activate MAP kinase by phosphorylating first a tyrosine residue followed by a threonine residue. A number of studies indicate that G protein-coupled receptors are also capable of activating the MAP kinase cascade by both ras-dependent and -independent pathways (Robbins et al., 1992; Alblas et al., 1993; Howe and Marshall, 1993; Winitz et al., 1993; Seufferlein et al., 1995). In addition, vasopressin has been found to activate p42 MAP kinase in vascular smooth muscle cells (Kribben et al., 1993). Using an antibody that recognizes tyrosine-phosphorylated p42 (ERK1) and p44 MAPK (ERK 2) we have demonstrated that vasopressin and a V1 agonist can stimulate tyrosine phosphorylation of MAPK in MCF-7 breast cancer cells. These data indicate that vasopressin is able to activate intracellular signaling events related to cell growth. These findings collaborate in vitro (Taylor et al., 1990) and in vivo studies (Chooi et al., 1994) which indicate that vasopressin is mitogenic for breast cancer cells. The role of vasopressin in breast cancer cells and its significance to tumor growth is becoming elucidated. These studies are expected to lead to new and rational approaches for the successful treatment of this disease.

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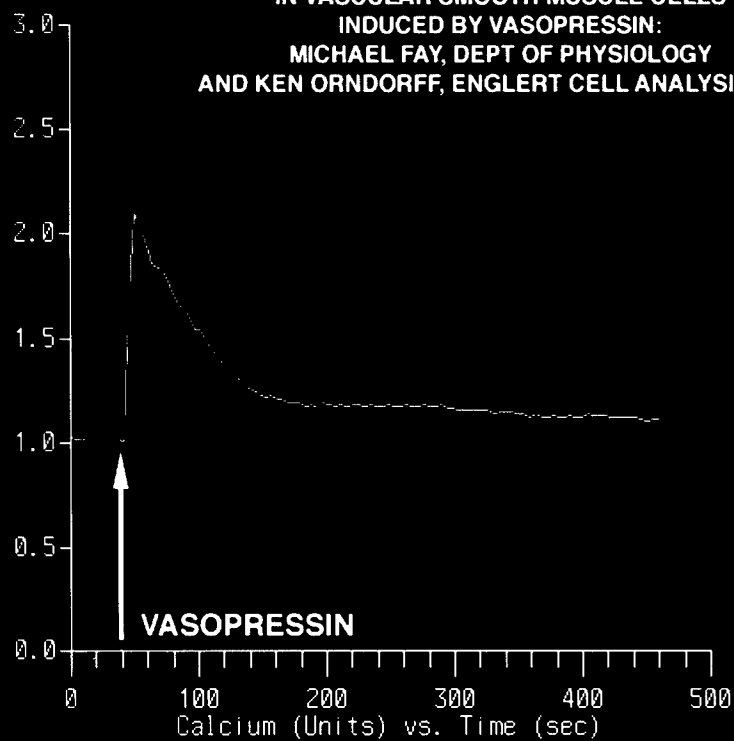
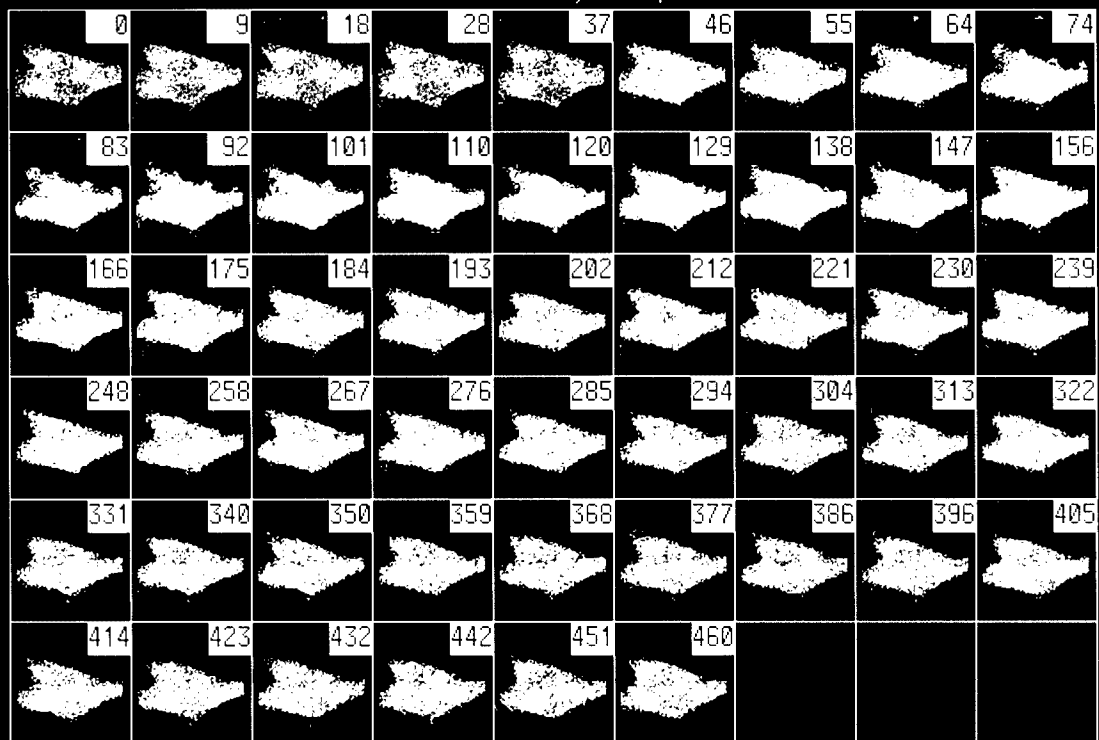Woods, D.J. and Monaco, M.E., Mol. Endocrinol., **2** 350 (1988).

(9) APPENDICES

The following items are located in the appendices:

1. One glossy print of vasopressin-induced calcium mobilization in A10 vascular smooth muscle cells.

2. An abstract to be presented at the 1996 Annual meeting of the American Society for Cell Biology.

3. A prepublication entitled Immunohistochemical evaluation of vasopressin gene expression in fibrocystic breast disease.

4. A reprint entitled Ionic signals in T47D human breast cancer cells.

CALCIUM FLUX
IN VASCULAR SMOOTH MUSCLE CELLS
INDUCED BY VASOPRESSIN:
MICHAEL FAY, DEPT OF PHYSIOLOGY
AND KEN ORNDORFF, ENGLERT CELL ANALYSIS LAB

Calcium (Units) vs. Time (sec)

A10 vascular smooth muscle cells, vasopressin added at 40 sec

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1996 STANDARD ABSTRACT FORM

EVIDENCE FOR THE EXPRESSION OF A NOVEL VASOPRESSIN -ACTIVATED CALCIUM MOBILIZING RECEPTOR (VACM-1) IN HUMAN BREAST CANCER AND LUNG CANCER ((K.A. Longo, M.J. Fay, J. Du, and W.G. North)) Department of Physiology, Dartmouth Medical School, Lebanon, NH 03756

The purpose of this study was to determine if a human homologue of the rabbit VACM-1 receptor is expressed in human cancer cells. Research indicates that vasopressin may be involved in human breast cancer and lung cancer pathophysiology, as an autocrine/paracrine factor. Vasopressin can act through four classes of receptors: V2, V1a, V1b, and the recently cloned VACM-1, a structurally unique member of this group that contains a single transmembrane domain. Vasopressin induced an increase in intracellular free Ca^{2+} in the breast cancer cell lines MCF-7, T47-D, and ZR-75 as well as in the lung cancer cell line H-146. RNA from these cell lines, as well as normal human tissues (kidney and lung), was used for reverse transcription polymerase chain reaction (RT-PCR) and Northern blot analysis. RT-PCR, using two primer sets designed against the rabbit VACM-1 sequence, amplified bands of the predicted sizes of ~674 bp and ~193 bp in all cell lines and tissues tested. Direct sequencing of PCR products obtained from MCF-7 and H-146 revealed a high degree of identity to the cloned rabbit VACM-1 cDNA sequence. Northern blot analysis, using the 674 bp PCR product as a probe, revealed the presence of three distinct bands, of approximate sizes 3.5, 5 and 6.5 kilobases, in the cancer cell lines. In summary, we have demonstrated the presence of mRNA for a novel vasopressin receptor in human cancer cell lines and normal human tissues.

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Immunohistochemical evaluation of vasopressin gene expression in fibrocystic breast disease.

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Summary: We previously found that expression of the vasopressin gene is a common feature of human breast cancer. In the present study we examined 17 different cases of benign fibrocystic breast disease for vasopressin expression using immunohistochemistry and antibodies directed against vasopressin and vasopressin-associated glycopeptide. All cases examined were negative for vasopressin gene expression using these antibodies. These results suggest that vasopressin gene expression occurs as part of the carcinogenic process rather than being a marker of cellular proliferation in the breast.

Key Words: Vasopressin, fibrocystic breast disease, immunohistochemistry

Running Title: Vasopressin and fibrocystic breast disease

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Introduction

Although a number of risk factors have been identified as possible causative agents for breast cancer, the etiological origin of this disease remains obscure (Henderson, 1993). Among those conditions which are predisposing towards breast cancer are proliferative breast disease, particulary atypical ductal and lobular hyperplasia (Dupont and Page, 1985; London et al., 1992; Connolly and Schnitt, 1993; Dupont et al., 1993). Atypical hyperplasia is classified as a borderline lesion because it has some of the histological features of carcinoma *in situ*. Previously we found that expression of the vasopressin gene is a common feature of human breast cancer using immunocytochemistry and antibodies directed against different regions of the vasopressin precursor (North et al., 1995). These results lead to the possibility that vasopressin expression could either, be a marker of cellular proliferation, represent part of the oncogenic process, or be a recognizable feature of cancer progenitor cells in precancerous breast lesions. We have commenced efforts to examine these questions by performing an immunohistochemical evaluation for vasopressin gene-expression using archival material representing various fibrocystic breast lesions.

Materials and Methods
Tissues

Formalin-fixed biopsy specimens were obtained from 17 patients with various forms of benign breast disease who were examined between 1975 - 1984 at Dartmouth Hitchcock Medical Center (DHMC, Lebanon, NH). The cases included, 4

cases of fibrocystic disease without hyperplasia, 9 cases of fibrocystic disease with

ductal or lobular hyperplasia, and 4 cases of fibrocystic disease with atypical ductal

hyperplasia. Diagnosis from pathology reports was confirmed by examining

hematoxylin- and eosin- stained sections. These cases were followed for the

subsequent development of breast cancer. Formalin-fixed specimens of human

hypothalamus and pituitary were obtained from autopsies performed at DHMC.

Antibodies

Rabbit polyclonal antibodies directed against vasopressin and the 18 amino

acid C-terminal vasopressin-associated glycopeptide were prepared using

previously published methods (North et al., 1991; Friedmann et al., 1994). Antibody

purification involved ammonium sulfate precipitation of the immunoglobulin fraction,

and fractionation on a column of protein A Sepharose with pH gradient elution (pH 7.6 -

pH 3.0). Antibodies were obtained as a pH 4.0 subfraction, dialyzed and lyophilized.

Based on dilution trials, antibodies to vasopressin and vasopressin-associated

glycopeptide were used at concentrations of 11 ng/ml (1:2,000) and 190 ng/ml

(1:800), respectively. Protein concentrations were determined using differential

spectroscopy (Waddell, 1956).

Immunohistochemistry

Sections of 4 - 6 microns from each specimen of fibrocystic breast disease

were deparaffinised and stained for vasopressin and vasopressin-associated

glycopeptide using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA,

USA) and avidin-biotin complex (ABC) immunohistochemistry (Guesdon et al., 1979). Tissues were rehydrated by washing with xylene, descending concentrations of ethanol, and PBS (2 x 3 min, ambient temperature). Slides were blocked with 10% normal goat serum in PBS for 20 min at ambient temperature. The blocking solution was aspirated and sections incubated overnight at 4 °C with primary antibody diluted in PBS with 1.5% goat serum. Following incubation with primary antibody the slides were washed with PBS (2 x 3 min). Goat anti-rabbit biotinylated secondary antibody diluted in PBS containing 1.5% goat serum was applied at a concentration of 20 μg/ml for 30 min. Unbound secondary antibody was removed by washing 2 x 3 min with PBS, and endogenous peroxidase activity blocked using 3% hydrogen peroxide dissolved in absolute methanol (Streefkerk, 1972). After washing with PBS (3 x 5 min) , slides were incubated with the avidin-peroxidase complex (25 μg/ml) for 30 min at ambient temperature. Slides were washed with PBS (2 x 3 min), and visualization of bound complex was achieved by adding a solution of 3,3'diaminobenzidine (0.2 mg/ml in PBS with 0.03% hydrogen peroxide) for 2 - 5 min. Tissues were then counterstained with hematoxylin, dehydrated in ascending concentrations of ethanol, washed in xylene, and coverslipped using permount. Antibody specificity was insured by incubating negative controls with pre-immune rabbit serum fractionated, using protein A Sepharose, at pH 4.0.

Results

Positive immunohistochemical staining was obtained for vasopressin neurons in human hypothalamus and for neuronal terminals of these neurons in the posterior

pituitary with both antibody preparations (data not shown). Alternatively, negative staining was obtained with both of these antibodies in the 17 cases of benign breast disease. In several tissue sections staining of mononuclear cells was evident. Staining of sections with pre-immune rabbit serum resulted in a lack of staining. As demonstrated previously, Fig 1a demonstrates positive staining of an acetone-fixed infiltrating ductal breast cancer biopsy specimen with the vasopressin-associated glycopeptide antibody (North et al., 1995). In this section the breast cancer cells demonstrate intense staining, and the normal ducts of the breast are unstained. Figure 1b demonstrates a tissue section of fibrocystic disease with adenosis which showed no immunostaining with the antibody to vasopressin-associated glycopeptide. A case of atypical ductal hyperplasia is represented in Fig 1c which exhibited negative staining with the antibody to vasopressin . It should be noted that the nuclei of cells appear dark because the nuclear counterstain hematoxylin was used. Follow-up of the medical records revealed that three individuals, one from each classification group, subsequently developed breast cancer.

Discussion

There is an increasing body of evidence which supports a connection between vasopressin and breast cancer. There are two published clinical reports of patients with breast cancer presenting with the syndrome of inappropriate antidiuretic hormone secretion (Gupta et al., 1986; Howard et al., 1993). Both in vitro and in vivo studies support a connection between vasopressin and breast cancer. Several studies indicate that a cell line derived from a dimethylbenz(a)anthracene-induced rat

mammary tumor possesses functional V_{1a} vasopressin receptors (Monaco et al.,

1978; Monaco et al., 1980; Guillon,et al., 1986; Kirk et al., 1986; Woods and Monaco,

1988). Vasopressin was shown to have a growth-promoting influence on MCF-7

breast cancer cells, presumably through V_1 vasopressin receptors (Taylor et al.,

1990). Another study has demonstrated that vasopressin-induces a rise in

intracellular free calcium in a human breast cancer cell line (Bunn et al., 1992). In a

transgenic mouse model of breast cancer, ectopic vasopressin was found to stimulate

cancer growth, but did not influence the time to tumor onset (Chooi et al., 1994).

Using antibodies directed against various regions of the vasopressin

prohormone and the technique of immunohistochemistry we found that expression of

vasopressin gene-related products is a common feature of breast cancers (North et

al., 1995). In the present study it was found that the various cases of fibrocystic

disease did not exhibit staining with antibodies directed against vasopressin or

vasopressin-associated glycopeptide. These two antibodies had previously given

positive immunostaining with all of the breast cancer specimens examined. The

results obtained in the present study suggest that expression of vasopressin gene-

related products is associated with the carcinogenic process and not with benign

breast disease. These results also indicate that expression of vasopressin gene-

related products is not just a marker of proliferation in the breast as indicated by the

lack of staining of the tissue sections consisting of hyperplasia. Examination of

subsequent medical records for these patients indicated that 3 of these cases (1 case

of fibrocystic disease without hyperplasia, 1 case of fibrocystic disease with

hyperplasia, 1 case of fibrocystic disease with atypical hyperplasia) went on to develop breast cancer. Although the sample number is small these findings suggest that vasopressin gene-related products are not markers of premalignant lesions which will subsequently develop into breast cancer. Such findings are consistent with those obtained by Chooi et al. (1994) with MMTV-VP vasopressin transgenic mice. In these animals it was observed that vasopressin had no influence on normal mammary gland function and development, and did not cause the development of hyperplastic alveolar nodules and ductal hyperplasia. Taken together, these data are suggestive that vasopressin gene expression is not involved in benign breast disease, and is not a marker of preneoplastic changes in the breast. It would therefore seem to be a marker of the carcinogenic process in the breast. We are in the process of examining cases of carcinoma *in situ* to determine if vasopressin gene-related products are detectable at this stage of breast cancer.

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Figure 1   **a,** Positive staining for vasopressin-associated glycopeptide in a tissue section of infiltrating ductal breast cancer. Note the lack of staining in a normal structure, as indicated by the arrow (magnification = 147 x). **b,** Negative staining for vasopressin in a tissue section of fibrocystic disease with atypical ductal hyperplasia (magnification = 147 x). **c,** Negative staining for vasopressin-associated glycopeptide in a tissue section of fibrocystic disease with adenosis (magnification = 147 x).

Ionic Signals in T47D Human Breast Cancer Cells

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ABSTRACT. Increasing evidence that ion channels play a key role in the modulation of cellular mitogenesis led us to investigate the membranes of T47D human breast cancer cells to identify the ion currents present. We report here the results of voltage-clamp studies in the whole-cell configuration on isolated, non-synchronized single cells obtained from a ductal breast carcinoma. In these studies we identified an outward rectifying potassium current and a chloride current. The potassium current activated at potentials more positive than −40 mV, reached an average value of 1.4 nA, and did not inactivate with time. This current was sensitive to block by extracellular tetraethylammonium chloride (TEA, IC_{50} = 1 μM), was insensitive to charybdotoxin (CTX, IC_{50} = 7.8 μM), and was not diminished by repetitive pulses separated by 1 s. Rapid voltage-dependent inactivation of the current was demonstrated by tail current analysis. The current appeared calcium-insensitive. Application of hyperpolarizing pulses did not elicit an inward potassium rectifier current. Treatment with tetrodotoxin did not reveal the presence of an inward sodium current. The potassium current was increased by the presence of aspartate in place of chloride and in the presence of the chloride channel blocker 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS). We conclude that currents present in T47D breast cancer cells include a chloride current and a voltage-gated potassium outward rectifier. We suggest that the potassium current, either alone or in conjunction with potassium currents reported in different human breast cancer cell lines by others, may play a role in the modulation of the cell cycle. CELL SIGNAL 8;4:279–284, 1996.

KEY WORDS. Ion channels, Breast cancer, Potassium currents, Mitogenic signals

INTRODUCTION

An increasing body of evidence supports the hypothesis that potassium currents signal cellular proliferation [1]. Small-cell lung carcinoma cells (SCCL) treated with the K^+-channel antagonist 4-aminopyridine (4-AP) demonstrate an attenuated outward K^+ current coincident with a decrease in cell proliferation [2]. In MCF-7 breast cancer cells the potassium channel antagonists quinidine, glibenclamide, and linogliride inhibit cell proliferation and cause the accumulation of cells in the G_0/G_1 phase of the cell cycle [3]. This effect on MCF-7 cells was attributed to the putative activity of adenosine triphosphate (ATP)-sensitive potassium channels. This cell line, established from pleural fluid of a patient with adenocarcinoma, retains certain characteristics of differentiated epithelium, including the presence of estrogen receptors [4]. A calcium-activated potassium current has been identified in MCF-7 cells [5] that, although correlated with cell proliferation, was not considered obligatory for growth [6].

The T47D breast cancer cell line selected for this study, originally established from the pleural effusion of a patient with infiltrating ductal carcinoma [7], also displays characteristics of an epithelial origin. These cells possess receptors for estrogen [8, 9], progesterone [6, 10–12], calcitonin [13], and vitamin D [14, 15]. We selected this cell line for investigation because estrogen-resistant clones have been produced, and we aim to develop this line as a model for evaluating breast cancer progression [16–18]. In view of the previous studies that have linked ion channels with mitogenesis, we initiated this study to identify the whole-cell currents present in unsynchronized cells of the T47D human breast cancer cell line.

MATERIALS AND METHODS
Cell Culture

The T47D human breast ductal carcinoma cell line was obtained from the American Type Culture Collection (ATCC HTB 133, Rockville, MD), and maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 0.2 IU bovine insulin/ml (Sigma, St. Louis, MO) and 10%

fetal bovine serum (FBS; Hyclone Labs, Logan, UT). Cells received fresh growth medium or were subcultured (1:3 using 0.25% trypsin + 0.02% ethylenediamine tetraacetic acid [EDTA]) every 2–3 d.

Cell Preparation

Cells were plated onto glass coverslips approximately 18 h before patch-clamp studies were initiated. Each coverslip was placed in a chamber of 0.2 mL volume and washed with extracellular physiological solution. The chamber was placed on the stage of an inverted Nikon microscope and viewed with Hoffman optics (×640). Electrode preparation and other experimental details have been presented previously [19–21].

Solutions

The physiological external bath solution for recording whole-cell currents contained the following (in mM): 140 NaCl, 4.5 KCl, 2.0 CaCl$_2$, 1.0 MgCl$_2$, and 10 4-(2-hydroxy-ethyl)-1-piperazine-N'-2-ethanesulfonic acid (HEPES), pH balanced to 7.3 with NaOH. The pipette solution contained (in mM) 140 KCl, 1.1 ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.1 CaCl$_2$, 2.0 MgCl$_2$, and 10 HEPES, pH balanced to 7.3 with KOH. [Ionized Ca^{2+}] = 10^{-8} M. All experiments were done at room temperature (22°C). Extracellular KCl was increased by substituting KCl for NaCl in the bath solution. Extracellular Cl was reduced by substituting potassium aspartate for KCl in the bath. Intracellular [Ca^{2+}] was increased by changing pipette solution CaCl$_2$ to 1.08 mM [free Ca^{2+}] = 4×10^{-6} M.

Blocking Agents

Tetraethylammonium chloride (TEA; Sigma) was dissolved in extracellular solution and added in different concentrations directly to the bath. Charybdotoxin (CTX) from *Leiurus* venom was obtained from Alomone Labs (Jerusalem, Israel). The CTX was dissolved in 0.1% BSA, 100 mM NaCl, 10 mM Tris (pH 7.5) and 1 mM EDTA. This solution required the addition of 0.01% bovine serum albumin (BSA) to the bath. Because the bath total volume was 0.2 mL, replacement of the bath required only two drops of fluid. Replacement of the bath effected rapid and complete mixing of experimental solutions, as demonstrated by addition of a water-soluble dye. 4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), a recognized Cl$^-$-channel blocking agent, was added to the bath.

Data Acquisition and Analysis

Currents in the individual cells were measured by rupturing the cell membrane in the lumen of the patch electrode by suction. Patch-clamp data were obtained with a List-EPC 7 clamp circuit (Medical Systems, Greenvale, NY). Experimental protocols, details of which are presented in the figure legends, were written for PCLAMP software (Axon Instru-
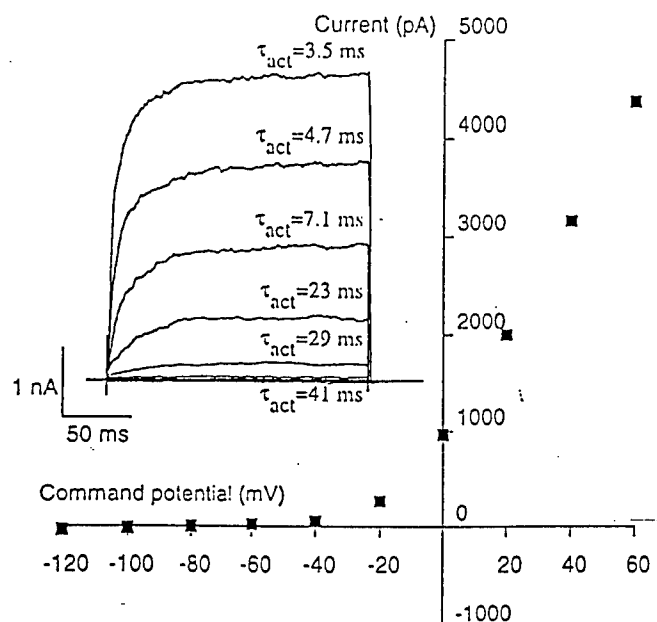

FIGURE 1. Outward rectifying K$^+$ current in T47D breast cancer cells. Current *vs.* voltage curve showing peak outward currents recorded in a cell held at − 80 mV and then stepped to voltages from − 120 mV to + 60 mV. Inset: Currents recorded from this cell. Note the lack of time-dependent inactivation. The time constant for activation (τ_{act}) was best described by a single exponential for voltage steps to − 40, − 20, and 0 mV, and by a double exponential at more positive depolarizations. The fast time constant is shown for each trace.

ments, Burlingame, CA). Data were digitized at 5 KHz and recorded with a 486 PC computer after filtering with an 8 pole Bessel filter at 1 KHz. Series resistance correction, capacity compensation, and leakage subtraction were done. All data are reported as mean ± S.E.M.

RESULTS

Patch-clamp recordings and analyses of T47D human breast cancer cells revealed the presence of a voltage-sensitive current. This current was identified as a voltage-gated, time-independent, TEA-sensitive, charybdotoxin-insensitive, outwardly rectifying potassium current, and was present in 71 of 79 cells studied. We found no evidence of an inward rectifying potassium current or of a sodium current.

Voltage-gated Potassium Current

After rupture of the cell membrane by suction, voltage steps were applied to the cell in a normal physiological ionic gradient from a holding potential of −80 mV to command potentials of − 120 mV to 60 mV. The large outward currents elicited are shown in the inset of Figure 1. The current was activated at −39.7 ± 2.2 mV (n = 10) and showed no time-dependent inactivation. At +40 mV depolarizations, the current reached a peak value of 1,376 ± 231 pA (n = 20). The time constants for activation could be represented by a single exponential at more negative voltage levels and
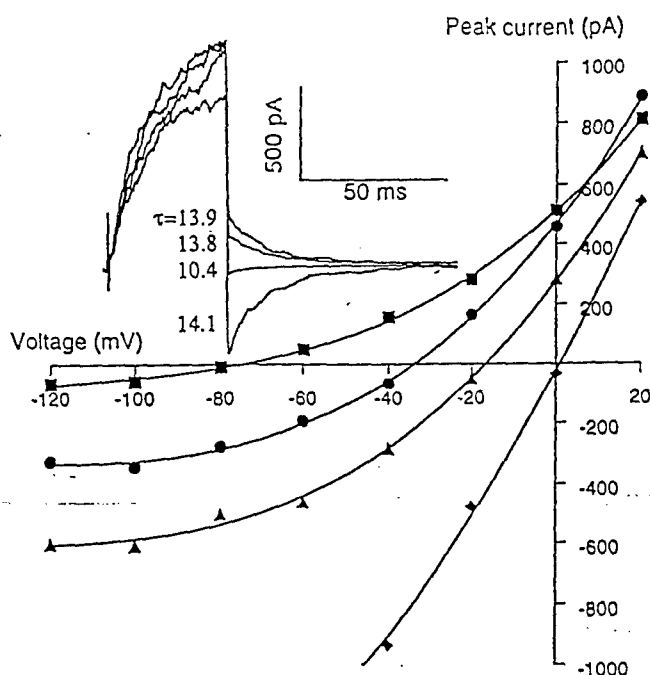
FIGURE 2. Tail currents. Peak current vs. voltage curves for tail currents recorded after a step depolarization from −80 mV to +40 mV. Pipette KCl concentration = 140 mM. External KCl concentration = 4.5 mM (squares), 38 mM (circles), 72 mM (triangles), or 140 mM (diamonds). Inset: Tail currents recorded at −20 mV in each external KCl concentration, with single exponential time constants (ms).

by a double exponential at more positive depolarizations. The fast time constants for activation are indicated for each current trace.

Tail Current Analyses

The outward current was activated by a voltage step from a holding potential of −80 mV to +40 mV. V_m was then stepped to voltages between +20 and −120 mV and tail currents were recorded as shown in Figure 2. Rapid voltage-dependent inactivation was observed. Changing bath KCl concentration produced changes in reversal potential for the outward current. In external KCl concentrations of 4.5, 38, 72, and 140 mM, E_{rev} was −84.5 ± 1.2 mV, −31.8 ± 1.7 mV, −17.6 ± 0.2 mV, and −2.6 ± 2.2 mV, respectively ($n = 7$). For these KCl concentrations, calculated E_{rev} for a K⁺-selective current was −86.6 mV, −32.8 mV, −16.7 mV, and 0 mV, respectively. Thus, this current appears to be carried by K⁺ ions. The rapid voltage-dependent recovery of the current is emphasized by the absence of use-dependent peak amplitude reduction when cells were repetitively depolarized at intervals of 1 s ($n = 8$).

Voltage Ramps

After holding the membrane potential at −80 mV, voltage ramps were applied to the cell membranes from −120 mV to +40 mV, with the resulting I vs. V graphs shown in
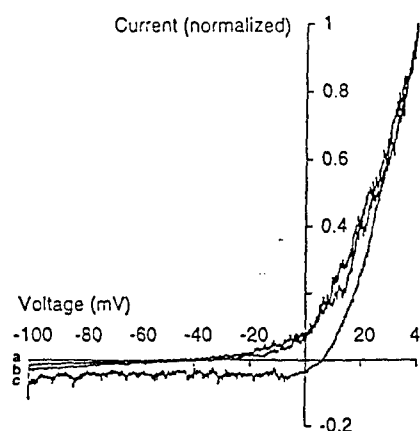

FIGURE 3. Voltage ramps in T47D breast cancer cells. From a holding potential of −80 mV, voltage ramps from −100 to +40 mV are shown. Curve a shows standard solutions. In curve b, pipette Ca²⁺ concentration was increased. The similarity between curves a and b suggests that no calcium-activated currents were present. Increasing external KCl concentration to 140 mM (c) alters the current vs. voltage relationships as expected. No evidence of an inwardly rectifying K current is seen, although the increase in leakage current suggests the presence of other non-voltage-activated currents.

Figure 3. These experiments were performed with standard pipette and bath solutions, and also after increasing pipette Ca²⁺ concentrations to levels as high as 4×10^{-6} and external KCl concentrations to 140 mM. In no case was a calcium-activated or inwardly rectifying current detected. However, the increased leakage current noted in symmetrical KCl solutions suggests the presence of additional K⁺ currents, perhaps a ligand-modulated current or the ATP-sensitive current reported by Woodfork et al. [3].

TEA

Figure 4 illustrates the results of 5 experiments in which voltage steps from −80 mV (holding) to +20 mV were applied in the presence of increasing concentrations of TEA chloride. Exponential curve fitting allowed calculation of a 50% inhibitory concentration (IC_{50}) of 1 μM TEA chloride, indicating a marked sensitivity of the current to this substance.

CTX

Charybdotoxin, a toxic component isolated from the venom of the scorpion Leiurus, has been shown in a number of studies to selectively block calcium-activated K⁺ channels and to have a strong blocking action on other types of K⁺ channels. Results of 5 experiments in which cells were depolarized after exposure to CTX are shown in Figure 5. Calculated IC_{50} was 7.8 μM, demonstrating an insensitivity of the current to the blocking effects of CTX. Both the solvent and BSA were tested in the absence of CTX and were found to have no effect on currents. The solution containing CTX completely replaced the bath in order to assure complete mixing.
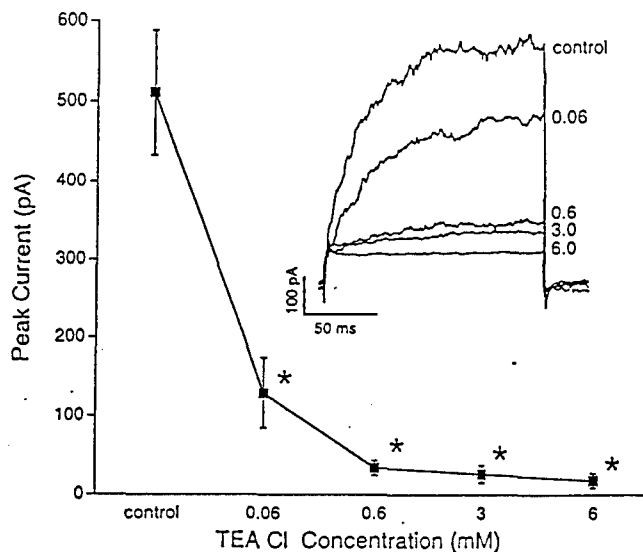
FIGURE 4. Effects of TEA chloride. Dose-related reduction in peak current following exposure to TEA chloride is demonstrated. Inset: Typical current recordings of a step depolarization from −80 mV to +20 mV after exposure to increasing TEA Cl concentrations. *$P < 0.0001$ vs. control by one-way ANOVA for repeated measures and Tukey's Honestly Significant Different Test.

Inward Currents

SODIUM. To determine whether a sodium inward current was present, we added TTX (3×10^{-6} M) to the bath solution. The membrane was subjected to a more negative holding potential to remove any inactivation that might be present, and the membrane was then depolarized by step pulses. No effect on the whole-cell current was detected, leading us to conclude that a voltage-gated sodium current is not present.

POTASSIUM. Experiments were designed to test for the presence of an inward potassium rectifier. The membrane
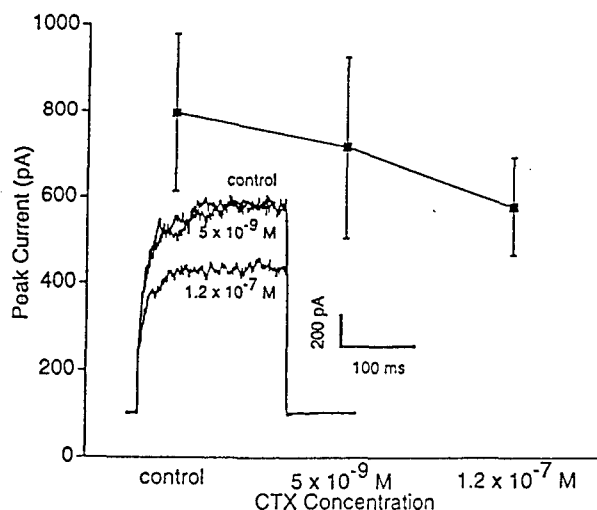

FIGURE 5. Effects of CTX. Increasing concentrations of CTX produce minimal decreases in peak outward current following a depolarization of −80 mV to +20 mV ($P = 0.11$). Inset: Typical current recordings after exposure to each concentration of CTX.
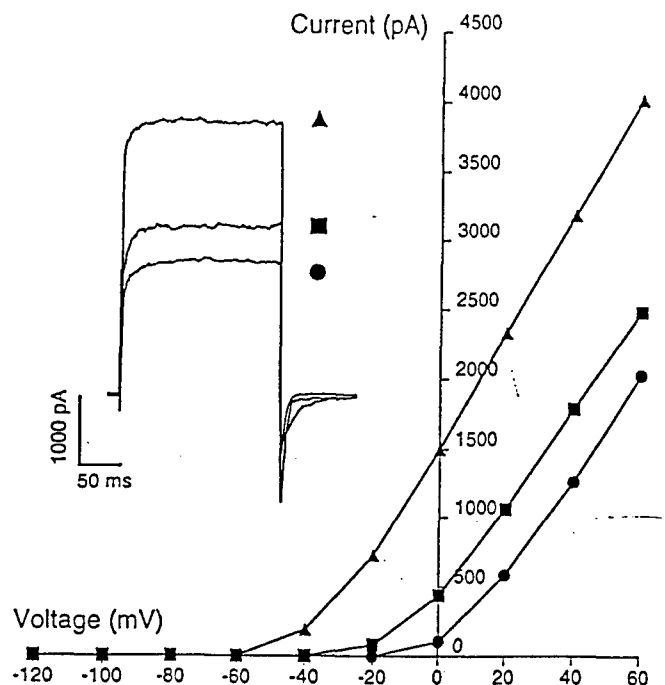

FIGURE 6. Augmentation of outward current by aspartate. From a holding potential of −80 mV, voltage steps from −120 to 60 mV were applied. The inset displays currents recorded after voltage steps to 60 mV in physiological bath solution (square), symmetrical 140 mM KCl solution (circle), and after substitution of 140 mM K aspartate for KCl (triangle). Current–voltage relationships for the same experiments demonstrate a marked increase in outward current after aspartate substitution.

was stepped to various hyperpolarized potentials in physiological, reversed, and altered extracellular potassium concentrations. There were no currents detected under these conditions.

CALCIUM. Increased amounts of calcium delivered both to the pipette and to the bath did not effect any changes in the outward current. With potassium replaced by CsCl in the pipette and with TEA in the bath, increased calcium in and out and addition of $BaCl_2$ (20 mM) did not reveal any inward current. Addition of CTX did not change the magnitude or time course of the potassium current. We conclude that calcium does not affect this voltage-gated potassium current.

CHLORIDE. The substitution of aspartate for chloride in the bath solution greatly enhanced the potassium current, as shown in Figure 6. The membrane potential was held at −80 mV, after which depolarizing steps were delivered from −120 mV to 60 mV. The marked increase in the total outward current is shown in the current traces and in the I vs. V relationship.

Chloride substitution experiments, tested by tail-current analyses, gave further evidence of the chloride component of the total current, as shown in Figure 7A.-D. The cell membrane was held at −80 mV and then depolarized to 40 mV. Tail currents were then measured as the membrane
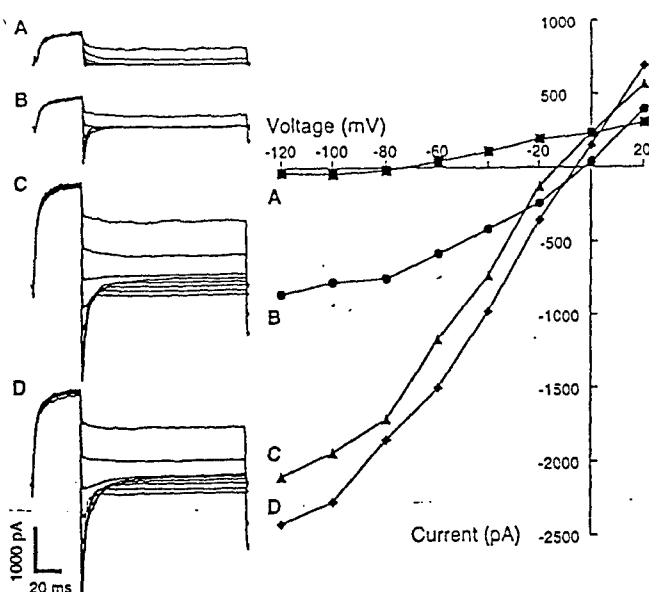
FIGURE 7. (A–D) Effects of chloride substitution on tail currents. Current traces A, B, C, and D display tail currents recorded after a depolarization to 40 mV from a holding potential of – 80 mV. Shown are tail currents produced by voltage steps from 20 to – 120 mV. The current-voltage curves for these traces are shown at the right. The pipette contained 140 mM KCl. Panel A was recorded in physiological bath solution. Panel B was recorded in symmetrical KCl solution and demonstrates a reversal potential near 0 mV. In C, 140 mM K aspartate was substituted for KCl in the bath solution and a change in reversal potential occurs. In D, 70 mM KCl with 70 mM K aspartate produced an intermediate change in reversal potential.

was stepped from 20 to – 120 mV in a normal physiological gradient (Fig. 7A). The potassium content of the bathing solution was then elevated so that the potassium gradient was symmetrical (Fig. 7B). Under this condition, the reversal potential was near 0 mV, (I vs. V graph), as one would predict for a potassium current. The reversal potential was then observed to shift as K^+ aspartate was substituted for KCl (Fig. 7C). The reversal potential again shifted along the voltage axis as the bath was changed to contain 70 mM KCl and 70 mM K aspartate (Fig. 7D).

An increase in the outward current was also measured when the chloride channel blocker DIDS was added to the bath. Application of a continuous ramp of increasing voltage (– 140 to 40 mV) from a holding voltage of – 80 mV resulted in an increase in the outward current from 1,042 pA to 1,265 pA, as shown in Figure 8. We conclude from these data that a chloride current is present in these cells.

DISCUSSION

The voltage-gated potassium current we have characterized in T47D breast cancer cells activates rapidly at a membrane voltage of – 40 mV, is outwardly rectifying, and displays no time-dependent inactivation. Peak currents are very large and at a depolarization level of 40 mV (holding: – 80 mV), reached levels of 1.4 nA. This current is sensitive to block
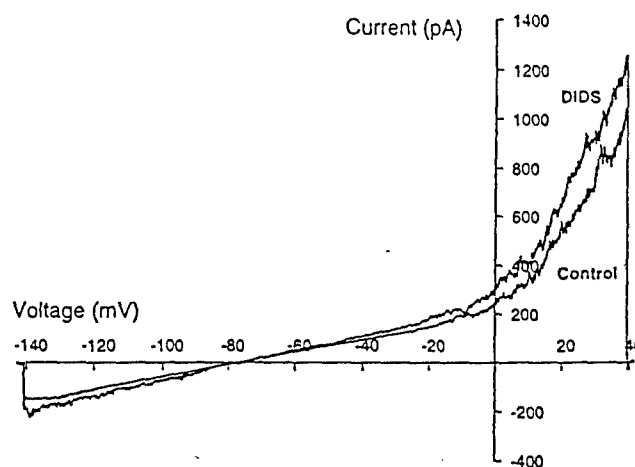

FIGURE 8. Increase in outward current after addition of DIDS. From a holding potential of – 80 mV, a continuous ramp of increasing voltage from – 140 mV to 40 mV was applied. The pipette contained 140 mM KCl. During the control recording, the bath contained standard physiological solution. After the addition of DIDS 4×10^{-4} M to the bath, peak current increased from 1,042 pA to 1,265 pA. This suggests that DIDS blocked an inward Cl^- current.

by TEA but not to CTX. TEA is a widely documented blocker of a potassium channels, but CTX is now known to block other potassium channels as well as the calcium-activated potassium channels [22]. In some cells, CTX blocks even the voltage-gated maxi-K channel [23]. In the experiments reported here, increased levels of internal calcium did not effect any change in either the magnitude of the potassium current or in the reversal potential, results that indicate that a calcium-activated potassium current is either not present or is of such relative magnitude compared to the outward rectifier that it is not readily visible by the whole-cell technique. In experiments in which the membrane holding potential was increased to hyperpolarized levels of – 140 mV, no evidence of an inward rectifier was seen. The reversal potentials measured in a range of trans-membrane potassium concentrations compared with the calculated values provide clear evidence for the ionic identity of the charge carrier. This current was not changed in the presence of tetrodotoxin, an observation that supports our conclusion that a sodium current is not present.

This report documents the first recordings of electrical activity from the T47D cell line of human breast cancer, an epithelial-like cell line obtained from an infiltrating ductal carcinoma of the breast. Another epithelial-like cell line of human breast cancer (MCF-7), obtained from an adenocarcinoma, has been explored by Woodfork et al. [3]. They concluded, from experiments using known potassium blockers on cell proliferation, that a ligand-activated potassium channel (i.e., an ATP-sensitive channel) is most likely involved in the proliferation of MCF-7 cells. In a preliminary report of voltage-clamp experiments [24], three types of current/voltage relations were described in MCF-7 cancer cells. In the presence of Mg-UDP (uridine diphosphate to enhance activation of K_{ATP}) in the pipette, linear I vs. V relationships

showed reversal potentials of −62 mV and −7 mV, respectively, while a third current was outwardly rectifying and reversed at −23 mV. Only the third type was observed when 2 mM ATP was also added to the pipette. The only current reported that appears similar to the outward rectifier we describe in T47D cells is a linear current with a reversal potential of −62 mV. The major potassium current in these two cell lines seems to be remarkably-different. While we have not yet specifically studied the effects of ATP, we did note an increased leakage current in symmetrical potassium, which suggests the presence of an additional component.

Evidence is presented here for the presence in T47D cells of a chloride channel. The data include enhancement of the outward current by aspartate substitution for chloride, enhancement of the outward current in the presence of the chloride blocking agent DIDS, and the shift of the reversal potential in the presence of altered chloride concentrations in the bath. These data are consistent with the criteria for the identification of a chloride current.

In view of the complex nature and behavior of breast cancer cells, it is not difficult to appreciate that chloride ions may function in multiple roles (e.g., membrane stabilization, regulation of intracellular pH, modulation of secretion, and cell volume control). These cells exhibit prolific secretory behavior. Another possibility to be considered in regard to this chloride current is that the aspartate itself may unmask a different potassium component, since a variety of amino acids are known to exert excitatory effects on cells that have receptors for these agents [25]. Further studies should help to characterize and identify this component of the whole-cell currents.

Whether the apparent differences in the currents measured in the MCF7 and T47D cell lines are related to some basic property of these two types of cells or to variations in the stage of the cell cycle may be resolved in future studies using cell-cycle-synchronized cultures. Based on previous studies with other cell types [1], we suggest that it is more likely that the potassium current will be involved in modulation of the cell cycle while the chloride channel will be linked to secretion, regulation of cell volume, or some other cellular function.

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MCMR-RMI-S   (70-1y)   10 Aug 98

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